CDP-diacylglycerol synthases regulate the growth of lipid droplets and adipocyte development

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Abbreviations: CDS, CDP-diacylglycerol synthases; CL, cardiolipin; ER, endoplasmic reticulum; GFP, green fluorescent protein; HILIC, hydrophilic interaction liquid chromatography; LDs, lipid droplets; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIS, phosphatidylinositol synthase; PPARγ, peroxisome proliferator-activated receptor-γ; TAG, triacylglycerol; TLC, Thin Layer Chromatography.
ABSTRACT

The expansion of lipid droplets (LDs) and the differentiation of preadipocytes are two important aspects of mammalian lipid storage. In this study, we examined the role of CDP-diacylglycerol (CDP-DAG) synthases (CDS), encoded by \textit{CDS1} and \textit{CDS2} genes in mammals, in lipid storage. CDS enzymes catalyze the formation of CDP-DAG from phosphatidic acid (PA). Knocking down either \textit{CDS1} or \textit{CDS2} resulted in the formation of giant or supersized LDs in cultured cells. Moreover, depleting \textit{CDS1} almost completely blocked the differentiation of 3T3L1 preadipocytes, whereas depleting \textit{CDS2} had a moderate inhibitory effect on adipocyte differentiation. The levels of many phosphatidic acid (PA) species were significantly increased upon knocking down \textit{CDS1}. In contrast, only a small number of PA species were increased upon depleting \textit{CDS2}. Importantly, the amount of PA in the endoplasmic reticulum was dramatically increased upon knocking down \textit{CDS1} or \textit{CDS2}. Our results suggest that the changes in PA level and localization may underlie the formation of giant LDs as well as the block in adipogenesis in CDS-deficient cells. We have therefore identified CDS1 and CDS2 as important novel regulators of lipid storage, and these results highlight the crucial role of phospholipids in mammalian lipid storage.

SUPPLEMENTARY KEYWORDS

endoplasmic reticulum, lipin-1, PPARγ, phosphatidic acid, phosphatidylcholine, phosphatidyglycerol, phosphatidylinositol, phosphatidylinositol synthase, triacylglycerol
INTRODUCTION

Lipid droplets (LDs) are highly dynamic organelles that play a central role in mammalian energy storage. LDs are also involved in many cellular functions, including protein storage and degradation, membrane and lipid trafficking (1-4). Each LD contains a hydrophobic neutral lipid core enclosed by a phospholipid monolayer (5). In mammals, the lipid core comprises mainly triacylglycerols (TAGs) and cholesteryl esters. The molecular events underlying the biogenesis of LDs remain to be determined (6). While several models have been proposed, the prevailing view is that LDs originate and bud from the endoplasmic reticulum (ER), followed by expansion and maturation (7). A recent study suggests that the fat storage-inducing transmembrane proteins are required for proper budding of LDs from the ER (8). The growth of LDs is not fully understood as the size of LDs varies within different tissues and even within the same cell type (9). Recent studies have established the Cide family proteins, Cidec/FSP27 in particular, as key regulators of LD growth/fusion in adipocytes (10, 11). Phospholipids, especially phosphatidylcholine (PC) and phosphatidic acid (PA), also appear to be important regulators of LD growth and proliferation as a decrease in PC or an increase in PA can both result in the formation of giant or “supersized” LDs (1, 9, 12, 13). LDs can also grow by lipid synthesis in situ as TAG synthesis enzymes can relocate from the ER to LD surface to mediate LD growth (14). Despite these important findings, our understanding of LD biogenesis and growth is still at its infancy, and additional mechanisms and regulators remain to be identified.

Changes in the cellular dynamics of LDs are associated with human metabolic disorders such as obesity, atherosclerosis and hepatic steatosis (15). The development of obesity is characterized by the accumulation of enlarged adipocytes loaded with giant LDs, which is due, at least in part, to enhanced LD expansion at the cellular level and adipocyte differentiation at the systemic level (16). Therefore, understanding the molecular mechanisms underlying the expansion of LDs and adipocyte development will provide insights into how therapeutic strategies can be developed against human metabolic diseases.

To identify novel gene products regulating the cellular dynamics of LDs, we carried out genome-wide screens in the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*). Our initial screen identified Fld1p (yeast homologue of human SEIPIN) as a major regulator of LD growth. Fld1p/SEIPIN has been
implicated in the metabolism of fatty acids and phospholipids (13, 17-19), as well as in TAG synthesis (20). Importantly, SEIPIN is also essential for adipocyte differentiation (13, 19, 21-23). Therefore, SEIPIN can regulate both cellular and systemic lipid storage. In subsequent genome-wide screens, we identified a number of additional gene products in yeast that impact LD growth. This includes CDS1, which encodes a CDP-diacylglycerol synthase (CDS) on the ER. Knocking down CDS1 leads to formation of “supersized” LDs in yeast (13). In mammals, there are two CDS enzymes: CDS1 and CDS2 (24). CDS enzymes catalyze the formation of CDP-DAG from PA, the precursor for all phospholipids and TAG synthesis. CDS1 and CDS2 are believed to localize to the ER, where they regulate the synthesis of phosphatidylinositol (PI) and phosphatidylglycerol (PG) (25). Although the biochemical functions of CDS1 and CDS2 have been characterized, little is known about their involvement in cellular lipid storage (LD formation) and adipocyte differentiation. Here, we show that, like SEIPIN, CDS1 and CDS2 regulate both LD expansion and adipocyte development, most likely through modulating the level of PA on the ER.
MATERIALS AND METHODS

Cell Culture, Transfection, and Treatments HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), while 3T3-L1 preadipocytes were maintained in the same medium but containing 10% newborn calf serum (NCS) instead of FBS. Transient transfections of small interfering RNA (siRNA) were performed at 20 nM using Lipofectamine RNAiMAX reagent (Life Technologies) according to manufacturer's instructions. SiRNAs against human or mouse CDS1 and CDS2 were purchased from Sigma Aldrich and Shanghai GenePharma Co. Ltd (Table S1). Transient plasmid transfections were performed using Lipofectamine LTX plus reagent (Life Technologies), consistent with the manufacturer’s instruction. ECFP-CDS1 was a gift from Dr. Beatriz Caputto; pmGFP-PASS, Rab10-mCherry, and GFP-PIS were generously provided as gifts from Drs. Guangwei Du and Gia Voeltz. Oleate (Sigma) was dissolved in 0.1 M NaOH, by heating at 90 ºC for 10 min, and then prepared as stock solution containing 5 mM oleate coupled with 5% fatty acid-free bovine serum albumin (BSA; Sigma). To induce LD formation, the stock oleate solution was added into cell culture at indicated concentrations for a desired time period. To differentiate into adipocytes, 3T3-L1 preadipocytes were grown for 2 days after confluence. The medium was then changed to DMEM containing 10% FBS, 1% P/S, 5 µg/ml insulin, 1 µM dexamethasone (DXM), and 0.5 mM isobutylmethylxanthine (IBMX). After 2 days, DXM and IBMX were withdrawn, and insulin was kept in culture for additional 2 days. Adipocytes were fully differentiated on day 8 after differentiation. Transient siRNA transfection was performed in preadipocytes at 50% confluence, and reperformed 1 day prior to inducing differentiation.

Fluorescence Microscopy Cells grown on coverslips were fixed with 4% paraformaldehyde (PFA; ProSciTech) for 15 min at room temperature (R.T.), followed by permeabilization with 0.1% Triton X-100 (Sigma) for 15 min. The LDs were stained with either BODIPY 493/503 (Life Technologies), Nile Red (Sigma), or HCS LipidTOX™ Neutral Lipid Stains Deep Red (Life Technologies). In BODIPY staining, fixed cells were incubated with 2 µg/ml dye in 150 mM NaCl for 10 min at R.T. in dark. Nile Red powder was dissolved in acetone to make 0.5 mg/ml stock solution, which was then further diluted to 200 ng/ml in prechilled 75% glycerol, and applied to cell staining for 45 min at R.T. in dark. LipidTOX
was applied for cell staining at 1:1,000 dilution for 45 min at R.T. in dark. The mitochondria were stained with MitoTracker® Red FM (Life Technologies) at 1:10,000 dilution for 45 min at R.T. in dark. The ER was stained with an indirect immunolabeling procedure. Fixed cells were blocked with 3% BSA for 1 h, and then incubated with rabbit anti-Calnexin (#2433, Cell Signaling Technology) at 1:50 dilution in 3% BSA overnight at 4 °C, followed by incubation with Alexa Fluor® 594 conjugated goat anti-rabbit secondary antibody (1:500; Life Technologies) for 1 h at R.T. in dark. Cells were viewed using Olympus FV1200 confocal microscope. 3D rendering was carried out using Huygens Essential Software (Scientific Volume Imaging). The diameters of the LDs were measured using ImageJ software (NIH).

**Western Blots** Cells were harvested in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride) containing additional commercial protease inhibitors (cOmplete™ ULTRA tablets; Roche) and phosphatase inhibitors (PhosSTOP tablets; Roche), and lyzed using bioruptor (Diagenode). Proteins were quantified by bicinchoninic acid (BCA) assay (Sigma), and then subjected to electrophoresis on Bis-Tris gels under denatured and reducing conditions. Immnoblots were performed with the following primary antibodies: anti-CDS1 (#ab88121), anti-RFP (#ab65856), anti-phospho-IRE1α (#ab124945) (Abcam); anti-total-IRE1α (#3294), anti-phospho-eIF2α (#3597), anti-total eIF2α (#2103), anti-CHOP (#5554), anti-Calnexin (#2433) (Cell Signaling Technology); anti-β-actin (#P2103) (Sigma); anti-CGI-58 (#12201-1-AP) (ProteinTech).

**Phospholipid Extraction and Mass Spectrometry** Phospholipids were extracted following Folch’s protocol (26). HeLa cells grown in 10cm dishes were collected by scraping into PBS. Aliquots were preserved to determine the protein concentrations by BCA assay. Cells were pelleted by centrifugation at 800g for 5 min, and then homogenized with 6 ml HPLC grade chloroform/methanol (2:1). The internal standards of phospholipids including PA 17:0/17:0, PC 19:0/19:0, phosphatidylethanolamine (PE) 17:0/17:0, PG 17:0/17:0, and PI 17:0/20:4 were added prior to rocking for 15 min at R.T., followed by addition of 750 µl of ultra-pure water (Life Technologies). The mixture was spun down for 5 min at 1500g. The lower phase was transferred to a new glass tube, followed by addition of 1.2 ml of ultra-pure water, briefly vortexed, and spun at 800g for 10 min. The lower phase containing phospholipids was
transferred to glass vials and air-dried. Dried samples were reconstituted in 100 µl of chloroform/methanol (2:1), and subjected to HPLC-MS/MS using Q-Exactive Mass Spectrometer. The phospholipids were separated by hydrophilic interaction liquid chromatography (HILIC) using 5 mM ammonium formate and 0.1% formic acid, acquired using both postitive and negative electrospray ionization mode, and analyzed using Lipid Search software.

Neutral Lipid Extraction and Thin Layer Chromatography HeLa cells grown in 10cm dishes were washed in PBS, and lysed with 0.1 M NaOH with rocking for 10 min at R.T.. Then 0.1 M HCl was added to the culture dishes. The cell lysates were transferred to 15 ml tubes and the protein concentrations were determined by BCA assay. To the cell lysate, 2 ml of methanol was added, followed by 2 ml of hexane (adapted from(27)). The mixture was vortexed for 30 s and spun at 1000g for 5 min. The top layer was transferred to a glass vial and air-dried. The neutral lipids were reconstituted in hexane, separated by Thin Layer Chromatography (TLC) using Silica Gel 60 plate (Millipore), and developed in a solvent system consisting of hexane/diethyl ether/glacial acetic acid (85:15:1). The lipids were stained with iodine vapors. The TLC plate was scanned using the EPSON PERFECTION 4490 PHOTO, and the TAGs were quantified using ImageJ software.

Lipid Droplet (LD) Isolation The LD isolation was performed following the protocol as described in (28). HeLa cells transfected with siRNA for 48 h prior to the treatment with 200 µM oleate for additional 16 h. Cells were harvested in PBS, and pelleted at 1,000g for 10 min. Then cells were resuspended in 10 ml of prechilled buffer A containing 20 mM tricine and 250 mM sucrose (pH 7.8, adjusted with KOH). Cell suspension was incubated on ice for 20 min. Dounce homogenizer was used to disrupt cells by gentle strokes for 25 times. The homogenate was centrifuged at 3,000g for 10 min at 4 °C to remove the nuclei, cell debris and unbroken cells as pellet, and the supernatant was preserved as post-nuclear supernatant (PNS). The PNS was transferred to the ultracentrifuge tube, followed by addition of 2 ml of buffer B containing 20 mM HEPES (pH 7.4), 100 mM KCl, and 2 mM MgCl₂. The centrifugation was then performed at 182,000g for 45 min at 4 °C using P40ST rotor on Hitachi HIMAC Ultracentrifuge. After ultracentrifugation, pellet was kept as total membrane (TM) fraction, solution from the middle of the gradient was saved as cytosolic (Cyto) fraction, and the LDs from the top band of the gradient were
carefully collected. The TM and LDs were washed with buffer B. Protein extracted from all fractions was subjected to Western blots, and the phospholipids were extracted from LDs for lipidomic analysis.

**Oleate incorporation** To measure oleate incorporation, HeLa cells were treated with 1 µCi \(^{14}\)C-oleate for 30 min in a humidified tissue culture incubator. Neutral lipids were extracted and developed using TLC as mentioned above. The TLC plate was exposed to a BAS-MS imaging plate (Fujifilm) for 96 h, visualized using the FLA-5100 phosphorimager (Fujifilm), and quantified using ImageJ software.

**Oil Red O Staining** The fully differentiated 3T3-L1 adipocytes were fixed with 4% PFA for 1 h at R.T.. Dishes were washed twice with ddH\(_2\)O, followed by shaking in 60% isopropanol for 5 min at R.T.. Oil Red O (ORO; Sigma) was dissolved in 100% isopropanol to make 0.5% stock solution. The ORO working solution was made by mixing 6 ml of stock solution with 4 ml of ddH\(_2\)O. Adipocytes were stained with ORO working solution for 10 min, followed by 4 consecutive washes with ddH\(_2\)O. Dishes were scanned using UMAX scanner.

**RNA Extraction and qRT-PCR** Total RNA was extracted using Trizol™ reagent (Life Technologies) according to the manufacturer’s protocol. RNA concentration and purity were estimated using Nanodrop spectrophotometer (Thermo Fischer Scientific). The RNA was then reverse-transcribed into cDNA using Superscript VILO cDNA synthesis kit (Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed with a Rotor-Gene 6000 Real-Time PCR machine (Qiagen) using SYBR Green (KAPA Biosystems). Primers are listed in Table S2.

**Statistical Analysis** All data are expressed as mean ± S.D. Comparisons between two groups were analyzed by two-tailed \(t\)-test, while multiple groups were compared by one-way ANOVA, using GraphPad Prism 6.0 software. Differences at values of \(p<0.05\) were considered significant.
RESULTS

Down-regulation of CDP-DAG synthases results in the formation of “supersized” lipid droplets

To determine if CDP-DAG synthases in mammalian cells regulate the expansion/morphology of lipid droplets (LDs), transient siRNA knockdown experiments were carried out in HeLa cells and 3T3-L1 preadipocytes (Fig. 1). Compared to the control, CDS1 was downregulated by 81% and 48% in HeLa and 3T3-L1 cells, respectively; whereas CDS2 was downregulated by ~90% in both cell types. Notably, in siCDS2 HeLa cells, the mRNA expression of CDS1 was increased by 2.6 fold; but in siCDS1 cells, CDS2 level was unchanged (Fig. 1A, B). The knockdown efficiency was confirmed using additional siRNAs (data not shown).

Fluorescence microscopy indicated that knocking down CDS1 or CDS2 in both HeLa and 3T3-L1 cells resulted in the formation of giant or “supersized” LDs (Fig. 1C). For the purpose of LD classification, we arbitrarily categorized LDs with diameters ≥2 µm as supersized LDs. The population of LDs with a diameter of 3~4 µm or >4 µm increased, whereas the diameter < 1 µm decreased in CDS1 or CDS2 deficient HeLa cells (Fig. 1D). Additional siRNAs were used to validate that downregulation of CDS1 or CDS2 formed supersized LDs (data not shown). 3D rendering of siCTRL, siCDS1, and siCDS2 cells confirmed the “supersized” phenotype, and further validated the difference between the volume of normal LDs and supersized LDs (Suppl Fig. 1A-C).

Supersized LDs emerge following LD clustering upon CDS1 depletion

To characterize the formation of supersized LDs in greater detail, we first examined the dynamic LD formation over 10 h treatment with oleate in HeLa cells. As shown in Fig. 2A, siCTRL cells exhibited only small LDs with no apparent change in distribution in response to 200 µM oleate. In contrast, LD clustering appeared as early as 4 h post-treatment in siCDS1 cells, while it could be observed, to a lesser extent, at 8 h in siCDS2 cells. In comparison to 200 µM, treatment with 400 µM oleate resulted in the formation of obviously larger LDs, indicating that LD volume was paralleled with oleate loading. The LD clustering phenotype in siCDS1 cells was further confirmed by 3D rendering (data not shown).

The dynamic change of LD size was then assessed by Gaussian distribution curves (Fig. 2B). The peak of curves representing the median diameter of LDs shifted along with the increase in oleate exposure
time, reflecting oleate-induced LD growth in a time-dependent manner. Notably, up to 6 h post-treatment
with 200 µM oleate, mean value of LD diameters were approximately 0.43 µm in all three groups;
however, in the following 4 h, it increased by 14%, 62%, and 87% in siCTRL, siCDS1 and siCDS2 cells,
respectively. Meanwhile, treatment with 400 µM oleate almost doubled the mean LD diameter from 0.57
µm to 1.01 µm between 6 h to 10 h in siCTRL cells, whereas it drove the formation of supersized LDs
with a mean diameter of 1.83 µm and 1.43 µm at 10 h in siCDS1 and siCDS2 cells, respectively.

Localization of CDS1 and CDS2

The ER plays a major role in membrane lipid synthesis and in LD biogenesis and growth. A recent study
showed that Rab10 localizes to novel dynamic ER-associated structures that mark the leading ends of
newly growing ER tubule (29). Importantly, phosphatidylinositol synthase (PIS), the ER enzyme that
mediates the conversion of CDP-DAG to phosphotidylinositol, was found to localize to this Rab10
domain, and PIS functions immediately after CDS. CDS1 and CDS2 are known as ER enzymes
catalyzing the synthesis of CDP-DAG to produce eventually PI, PG, and CL, but it is unclear if they
localize to different ER domains. Thus we wondered if either one or both CDS enzymes may co-localize
with Rab10/PIS. We were also interested in the spatial relationship between LDs and the Rab10 domain
as it is not known if LDs originate from or interact with specific ER domains. To this end, we co-
transfected GFP-PIS with mCherry-Rab10, mCherry-CDS1, or CDS2-mCherry, and immunolabeled the
ER using antisera against calnexin in HeLa cells (Fig. 3A). Fluorescence microscopy revealed that PIS
only partially co-localized with calnexin, but it almost completely co-localized with Rab10. In contrast,
although the overexpressed CDS1 and CDS2 were diffused in the cytoplasm, they poorly co-localized
with PIS. To examine if LDs closely interact with the Rab10/PIS domain, we stained LDs with LipidTox
following transient transfection with GFP-PIS. Fluorescence microscopy indicated that the Rab10/PIS-
associated ER structures and LDs rarely co-localized (Fig. 3B).

TAG synthesis is increased in CDS1/2 knockdown cells

As CDS is the enzyme required for converting PA to CDP-DAG, down-regulation of the CDS genes
could cause an accumulation of PA and DAG, which could affect the synthesis of TAG, as well as that of
PC and PE through the Kennedy pathway. In fact, the formation of supersized LDs in siCDS1 and siCDS2
cells could result from excessive TAG accumulation and/or a reduction in total phospholipids, in particular PC and PE (12). We therefore measured the rate of oleate incorporation into TAG, as well as steady-state levels of TAG. siCDS1 and siCDS2 cells both exhibited an increase in the rate of oleate incorporation into TAG by approximately 50% (Fig. 4A). In the absence of oleate treatment, siCDS1 and siCDS2 increased cellular TAG by 42% and 55% respectively; whereas only siCDS2 significantly increased steady-state levels of TAG in response to oleate (Fig. 4B). By lipidomic analysis, we further examined the cellular levels of PC, PE, PG, and PI (Fig. 4C; PC in Fig. 5C). As the enzymes catalyzing synthesis of CDP-DAG followed by PG and PI, down-regulation of CDS1 or CDS2 indeed led to a decrease in PG and PI level. Compared to relatively constant PG, PI was more sensitive in response to CDS1/2 knockdown. In contrast, no significant change in total PC and PE was detected.

**PA metabolism upon CDS1 or CDS2 depletion**

CDS enzymes synthesize CDP-DAG from PA, which is the precursor to the de novo synthesis of all major phospholipids. Previous studies have shown that this cone-shaped phospholipid possesses fusogenic properties, contributing to LD fusion and the development of supersized LDs in yeast (13). In the absence of oleate treatment, knocking down CDS1 led to a nearly 2-fold increase in total cellular PA (Fig. 5A). Of all PA species examined, some highly abundant species, such as 16:0, 18:0, 18:1, as well as some less abundant species, such as 14:0, 17:0, 17:1, and 27:6, were substantially increased by siCDS1 in untreated cells (Fig. 5B). In contrast, siCDS2 did not affect total PA level in untreated cells, but significantly increased PA level in response to oleate treatment (Fig. 5A). In contrast to PA as the intermediate in phospholipid synthesis pathway, PC level is much more abundant and constant. Indeed, neither total PC level nor PC species was profoundly changed upon CDS gene depletion (Fig. 5C, 5D).

As the measurement of total cellular PA can not reveal any subcellular changes, we performed lipidomic analysis of PA level in isolated LDs from oleate-treated HeLa cells (Fig. 5E, 5F). Notably, PA level in the LD fraction was significantly increased after knocking down either CDS1 or CDS2; whereas PC level in LDs were unchanged (Fig. 5F). We next examined the subcellular localization of PA using fluorescent labeling with GFP-PASS, a PA sensor that contains the repeat of the Spo20p51-91 domain fused to green fluorescent protein (GFP) specifically binding to PA (30). In the co-staining of the endogenous
ER marker calnexin, fluorescence microscopy revealed that PA exhibited a ring-like distribution surrounding the ER in control cells; whereas knockdown of CDS1 or CDS2 led to an enhanced colocalization of GFP-PASS and calnexin, suggesting an accumulation of PA in the ER (Fig. 5G). As CDS1 and CDS2 could also affect mitochondrial lipids, we tested whether PA accumulates in the mitochondria upon CDS depletion. We stained the mitochondria with MitoTracker, and there is no significant colocalization between PASS and the MitoTracker (Fig. 5H).

**CDS Overexpression inhibits LD expansion**

Because knocking down CDS1 or CDS2 formed supersized LDs, we wonder if overexpressing CDS enzymes may have an opposite effect. For this purpose, we transiently transfected HeLa cells with eCFP-CDS1 or CDS2-mCherry, and their protein expression was detected by immunoblotting with CDS1 and RFP (against mCherry) antisera, respectively (Fig. 6A, 6D). Fluorescence microscopy indicated that overexpression of eCFP-CDS1 or CDS2-mCherry resulted in a dramatic reduction of LD number and size in HeLa cells (as indicated by arrows), as compared to either the untransfected cells in the same field or empty vector (eCFP-N1 and mCherry-N1) overexpressed cells (Fig. 6B, 6E). The population of LDs with a diameter >2 µm decreased, whereas the diameter < 1 µm increased by CDS1 or CDS2 overexpression (Fig. 6C, 6F). In addition, CDS1 and CDS2 overexpression substantially decreased total cellular TAG level by 47% and 42%, respectively (Fig. 6G), which was consistent with the reduced LD number and size as observed in fluorescence microscopy (Fig. 6B, 6E).

**CDS overexpression inhibits LDs expansion in LPIN1 knockdown cells**

The data above suggest that CDS may modulate LD morphology through changing the level of PA on the ER and LDs. Besides CDS1 and CDS2, the PA phosphatase Lipin-1 can also metabolize PA by converting PA to DAG followed by TAG, PC and PE synthesis. Lipin-1, encoded by LPIN1, is a known regulator of adipogenesis (31). To further investigate how CDS may regulate LD size, HeLa cells were transfected with siRNA against LPIN1, which decreased LPIN1 mRNA level by 60% (Fig. 7A). Knocking down LPIN1 also resulted in the formation of supersized LDs (Fig. 7B). Notably, siLPIN1-induced supersized LD phenotype could be substantially reversed by overexpressing eCFP-CDS1 or CDS2-mCherry (as indicated by arrows), as compared to those cells with no CDS overexpression in the same
field (Fig. 7C). We next measured the total cellular TAG level (Fig. 7D). Single transfection with siLPIN1 resulted in a significant reduction of TAG content, indicating that the supersized LD phenotype in these cells is not caused by increased TAG. Notably, co-transfection with CDS1 or CDS2 in LPIN1 knockdown cells caused no further change in TAG, suggesting that CDS overexpression reversed LD phenotype not by modulating TAG content. We next examined the phospholipids content (Fig. 7E). siLPIN1 increased total PA in HeLa cells, and co-transfection of CDS with siLPIN1 did reduce PA as compared to siLPIN1 alone. The levels of PC and PE showed no significant decrease upon depleting LPIN1, while the PG level increased. Overexpression of CDS1 or CDS2 increased the level of PI in LPIN1 deficient cells.

**Down-regulation of CDS1 inhibits adipocyte differentiation**

Besides LD expansion, PA has also been implicated in adipocyte differentiation (32-34). We hypothesize that the accumulation of PA in the ER and nuclear membrane upon CDS1/2 depletion could inhibit adipocyte differentiation possibly by interfering with PPARγ function. Firstly, the mRNA levels of CDS1 and CDS2, along with adipocyte differentiation markers, such as PPARγ, aP2, C/EBPα, Pref1, and Caspase 3, were determined on day 0, 2, 4, and 6 during the differentiation of 3T3-L1 preadipocytes (Fig. 8A). CDS1 mRNA expression steadily increased by 6.3 fold during 6 days differentiation, while CDS2 mRNA level exhibited a 60% decrease on day2, followed by a recovery up to day 6. We next examined if knockdown of CDS1 or CDS2 influences on adipocyte differentiation. As shown in Fig. 8B, siRNA transfection substantially down-regulated CDS1 mRNA level during day 0 to 4 that is the determinant period in adipocyte differentiation. On day 4, knocking down CDS1 resulted in 65%, 71%, and 75% decrease in PPARγ, aP2, and C/EBPα level, respectively, indicative of an inhibition on adipocyte differentiation. In agreement, Oil Red O (ORO) staining further revealed that the adipocyte differentiation was almost completely blocked in siCDS1 cells on day 8 (Fig. 8C). In contrast, knocking down CDS2 led to statistically significant, but biologically marginal, changes in mRNA levels of PPARγ, aP2, and C/EBPα (Fig. 8D). In addition, there was no distinguishable difference between siCTRL and siCDS2 cells in ORO staining (Fig. 8E).

ER stress has been shown to inhibit adipogenesis *in vivo* via unfolded protein responses (UPRs) such as phosphorylation of eIF2α and induction of CHOP, but not phosphorylation of IRE1α (35),
whereas others demonstrate that these UPRs are required for adipogenesis both in vivo and in vitro (36). To interrogate if ER stress was involved in siCDS1/2-induced suppression of adipocyte differentiation, we examined a variety of UPRs on day 0 of differentiation (Fig. 8F). Knocking down of CDS1 or CDS2 resulted in the phosphorylation of IRE1α, in contrast, only siCDS1 induced phosphorylated eIF2α and CHOP. However, the intensity of siCDS1-induced UPRs was not comparable to that in cells treated with tunicamycin (Tm), a chemical ER stress inducer.
DISCUSSION

In this study, we examined the role of CDP-DAG synthase, a key enzyme in phospholipid metabolism, in cellular lipid storage as well as in the differentiation of adipocytes. Our data demonstrate that both CDS1 and CDS2 can regulate the expansion of LDs. Notably, CDS1, but not CDS2, appears to be essential for adipogenesis. Our data provide an intimate link between the expansion of LDs at the cellular level and the differentiation of adipocytes at the systemic level, and suggest that phospholipids, e.g. PA, may play an important role in the regulation of two seemingly disparate processes.

LDs have now been recognized as dynamic organelles that constantly change their size and number (9). However, the molecular mechanisms underlying the expansion or contraction of LDs remain to be fully elucidated. Recent studies have identified key proteins such as FSP27 in the growth of LDs in adipocytes, and have also revealed a role of phospholipids in determining the growth and size of the LDs (9, 10, 37). For instance, decreased cellular PC or increased PA can both lead to the formation of giant LDs, possibly involving FSP27-independent LD fusion. The CDP-DAG synthase is an important enzyme in phospholipid metabolism because it sits at a key branching point of phospholipid synthesis where it converts PA to CDP-DAG for PI and PG synthesis. Knocking down CDS1 or CDS2 is predicted to increase the amount of PA and reduce the amount of PI and PG. Indeed, PA did accumulate, especially in the ER (Fig. 5). The levels of PI and PG were decreased in CDS1 deficient cells (Fig. 4C). The impact of depleting CDS1 on the level of PI and PG is somewhat surprising: a previous study demonstrated that CDP-DAG synthase is probably not a determining factor in controlling the de novo rate of PI synthesis or in establishing the cellular PI content as overexpressing CDS1 does not increase the cellular levels of CDP-DAG or PI (24). Given the complexity in phospholipid synthesis pathways, additional studies are required to fully understand the effect of CDS1/2 depletion on cellular PI and PG synthesis.

In relation to the formation of supersized LDs upon knocking down CDS1 or CDS2, it appears that increased PA, but not decreased PI or PG may play a major role. PG is primarily localized to mitochondria whereas PI is not a fusogenic lipid. In addition, there are no drastic changes in the level of neutral lipids, PC and PE in these cells. Moreover, the critical role of PA in supersized LD formation is further supported when supersized LDs are formed upon LPIN1 knockdown (Fig. 7). Importantly,
overexpression of CDS1 or CDS2 can restore normal LD morphology in LPIN1 depleted cells, likely through reducing the level of PA. Notably, overexpressing CDS1 or CDS2 did not further decrease the amount of TAG in siLPIN1 cells. Together, although a role for other lipid species such as PI and its derivatives in supersized LD formation cannot be ruled out, our data strongly suggest that PA may be a key regulator of LD growth in mammalian cells, as it is in yeast and fly (13, 38).

The differentiation of preadipocytes requires a transcriptional cascade that ultimately leads to the activation of the master regulator of terminal adipogenesis: peroxisome proliferator-activated receptor-γ (PPARγ) (39). Depleting CDS1 has a strong inhibitory effect on adipocyte development, whereas depleting CDS2 had a moderate inhibitory effect, possibly due to the upregulation of CDS1 mRNA (Fig. 1E). How does CDP-DAG synthase regulate adipogenesis? Given the changes in phospholipid profile upon depleting CDS1 or CDS2, we hypothesize that PA may play a key role in this process as well. The master driver of adipogenesis, PPARγ, has a large ligand binding pocket that can be activated by various metabolites that originate from phospholipid and fatty acids (39). Despite the abundance of naturally occurring molecules that activate PPARγ in cell-based assays, the endogenous regulators of PPARγ that are of physiological importance remain poorly defined. The recent identification of cyclic PA as an antagonist of PPARγ adds another layer to the regulation of PPARγ activity (40, 41). Therefore, certain PA species could serve as direct antagonists of PPARγ. Alternatively, increased PA could affect post-translational modifications of PPARγ or could trap PPARγ into certain membrane domains, preventing its activation. ER stress may be another factor that contributes to a blockage of adipogenesis in siCDS1 cells where eIF2α and CHOP are specifically activated. Given the severe effect of siCDS1 on adipogenesis, it is likely that both ER stress and PPARγ inhibition may play a role.

CDP-DAG synthase is an evolutionarily conserved, key enzyme in phospholipid metabolism. There is one CDS gene in yeast or fly, but there are two CDS genes in mammals. CDS1 appears to be the main isozyme as knocking down CDS1 led to drastic changes in the level of total PA. CDS1 and CDS2 also appear to be differentially regulated as CDS1, but not CDS2 is up-regulated during adipocyte differentiation (Fig. 8). Since both CDS1 and CDS2 are known to localize to the ER, we wondered if the two isoforms localize to different ER subdomains. The recently discovered Rab10/PIS domain on the ER
is associated with active membrane growth and phospholipid synthesis, but none of the CDS isoforms seems to co-localize with the PIS/Rab10 subdomain despite the fact that PIS functions immediately after CDS (Fig. 3A). It is possible that the two isozymes may prefer different PA species as substrates since changes in different subsets of PA species were observed when CDS1 or CDS2 was knocked down (42). However, it remains possible that CDS1 and CDS2 localize to different subdomains on the ER.

In summary, we have discovered novel functions of a classic enzyme in phospholipid metabolism: the CDP-DAG synthase. Our results implicate PA as a possible key regulator of LD expansion and adipogenesis, and highlight the important cellular and developmental functions of phospholipids.
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REFERENCES


FIGURE LEGENDS

FIGURE 1. Knocking down CDS genes leads to the formation of supersized lipid droplets (SLDs)
HeLa cells and 3T3-L1 preadipocytes were transfected with negative control siRNA (siCTRL), siCDS1, or siCDS2 for 48 h, followed by oleate treatment for additional 16 h. A-B: mRNA expression levels of CDS1 and CDS2 were determined by qRT-PCR in HeLa cells (A) or 3T3-L1 preadipocytes (B) in the absence of oleate treatment. C: LDs were stained with BODIPY 493/503 in oleate-treated cells, and visualized using a confocal microscope. Bar = 10 µm. D: LD size distribution in HeLa cells were determined. Data were collected by measuring the diameter of at least 1,000 LDs using the ImageJ software. A and B: Data were expressed as mean ± S.D from at least three biological replicates. *, p<0.05; **, p<0.01; ***, p<0.001; vs. siCTRL.

FIGURE 2. Supersized LDs develop from clustered LDs
HeLa cells were transfected with negative control siRNA (siCTRL), siCDS1, or siCDS2 for 48 h, followed by the treatment with oleate (OA) at 200 or 400 µM for indicated time points. A: LDs were stained with BODIPY 493/503, and visualized using a confocal microscope. Bar = 10 µm. B: Diameters of BODIPY stained LDs were measured using the ImageJ software. The dynamic changes of LD size were analyzed by Gaussian distribution curves using GraphPad Prism software. Data were collected from at least 200 LDs in each treatment.

FIGURE 3. Subcellular localization of CDS1 and CDS2
A: Co-localization of PIS with ER marker, Rab10, CDS1 and CDS2. HeLa cells were co-transfected with GFP-PIS, mCherry-Rab10, mCherry-CDS1, and CDS2-mCherry for 48 h. Calnexin was labeled by immunofluorescence staining. B: Co-localization of PIS with LDs. HeLa cells were transfected with GFP-PIS for 48h, followed by the treatment with 200 µM oleate for 16 h. LDs were stained with LipidTox Deep Red. Co-localization was examined using a confocal microscope. Arrows indicate the co-localization spots.

FIGURE 4. Lipid profiles of CDS knockdown cells
HeLa cells were transfected with negative control siRNA (siCTRL), siCDS1, or siCDS2 for 48 h prior to the oleate (OA) treatment. A-B: Cells were pulsed with $^{14}$C-oleate for 30 min (A) or treated with oleate (OA) for additional 16 h (B). Neutral lipids were extracted, and separated on Thin Layer Chromatography (TLC). $^{14}$C-oleate incorporation was quantified using phosphorimager and ImageJ software. n=2 (A). Total cellular TAG level was quantified using ImageJ software. n=5 (B). C: Lipidomic analysis of phospholipids. Phospholipids were extracted in cells untreated or treated with OA for 16 h, subjected to HPLC-MS/MS, and analyzed using Lipid Search software. n≥4. Data were expressed as mean ± S.D. *, p<0.05; **, p<0.01; ***, p<0.001; vs. siCTRL. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

FIGURE 5. PA level upon depletion of CDS
HeLa cells were transfected with negative control siRNA (siCTRL), siCDS1, or siCDS2 for 48 h prior to the oleate (OA) treatment. A-D: Lipidomic analysis of cellular PA and PC. n≥3. A, C: phospholipids were extracted from cells untreated or treated with 200 µM oleate (OA) for additional 16 h. B, D: PA or PC species in untreated cells. E, F: Lipidomic analysis of phospholipids in the LD fraction. After treated with 200 µM OA for 16 h, LDs were isolated using density gradient ultracentrifugation. The post-nuclear supernatant (PNS), total membrane (TM), cytosolic (Cyto), and LD fractions were subjected to Western blots for a series of subcellular fraction markers, such as Calnexin, β-actin, and CGI-58, as the control of cross-contamination (E). Phospholipids were extracted from LD fraction. n=3 (F). G, H: HeLa cells were co-transfected with siRNAs and GFP-PASS for 48 h. In the fixed cells, the ER was immunofluorescently stained with antisera against calnexin, and the mitochondria were stained with MitoTracker. Fluorescent images were taken using a confocal microscope. Data were expressed as mean ± S.D. *, p<0.05; vs. siCTRL. PA, phosphatidic acid; PC, phosphatidylincholine.

FIGURE 6. CDS overexpression inhibits LD expansion
HeLa cells were transfected with eCFP-N1 and eCFP-CDS1 or mCherry-N1 and CDS2-mCherry for 48 h, followed by the treatment with oleate for 16 h. A, D: The protein lysates from untreated cells were subjected to Western blots against CDS1 (A), RFP (D), and GAPDH. B, E: LDs were stained with Nile
Red (B) or BODIPY 493/503 (E) in oleate-treated cells, and visualized using a confocal microscope. Bar = 10 µm. Arrows indicate the CDS-overexpressing cells. C, F: LD size distribution in HeLa cells was determined. Data were collected by measuring the diameter of at least 500 LDs using the ImageJ software. G: Cellular TAG level was determined by thin layer chromatography following neutral lipid extraction, and quantified using ImageJ software. Data were expressed as mean ± S.D. n=4. **, p<0.01; vs. siCTRL.

FIGURE 7. CDS overexpression inhibits LD expansion in LPIN1 knockdown cells

A, B: HeLa cells were transfected with negative control siRNA (siCTRL) or siLPIN1 for 48 h, followed by the treatment with 200 µM oleate for additional 16 h. mRNA expression level of LPIN1 was determined by qRT-PCR in the absence of oleate treatment. n=3 (A). LDs were stained with BODIPY 493/503 in oleate-treated cells, and visualized using a confocal microscope (B). C-E: Co-transfection of siRNAs with the plasmids encoding eCFP-CDS1 or CDS2-mCherry was performed in HeLa cells for 48h. Cells were then treated with 400 µM oleate for additional 8 h. LDs were stained with either Nile Red or BODIPY 493/503, and visualized using a confocal microscope. Bar = 10 µm. Arrows indicate the CDS-overexpressing cells (C). Cellular TAG level was determined in oleate-treated cells by thin layer chromatography following neutral lipid extraction, and quantified using ImageJ software. n=4 (D). Phospholipids were extracted from untreated cells, subjected to HPLC-MS/MS, and analyzed using Lipid Search software. n=4 (E). Data were expressed as mean ± S.D. *, p<0.05; **, p<0.01; ***, p<0.001; vs. CTRL. #, p<0.05; ##, p<0.01; vs. siLPIN1. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

FIGURE 8. Knocking down CDS1 inhibits adipocyte differentiation

A-E: 3T3-L1 preadipocytes were induced to differentiate after transfection with negative control siRNA (siCTRL), siCDS1, or siCDS2 for 48 h. A, B, D: mRNA expression levels were determined by qRT-PCR on day 0, 2, 4, and 6 of differentiation. C, E: On day 8 of differentiation, 3T3-L1 cells were stained with Oil Red O. F: 3T3-L1 preadipocytes were transfected with siRNAs for 72 h. Protein lysates were
subjected to Western blotting. Tunicamycin (Tm; 2 µg/ml) was used for indicated time points as positive control. Data were expressed as mean ± S.D. n=3. *, p<0.05; **, p<0.01; ***, p<0.001.
Figure 1. Knocking down CDS genes leads to the formation of supersized lipid droplets (SLDs)
Figure 2. Supersized LDs develop from clustered LDs

A

Untreated

+OA 200 µM

+OA 400 µM

2h

4h

6h

8h

10h
Figure 2. Supersized LDs develop from clustered LDs (Cont.)

B

+OA 200 µM

+OA 400 µM
Figure 3. Subcellular localization of CDS1 and CDS2

A

![Calnexin](image1)

![Rab10](image2)

![CDS1](image3)

![CDS2](image4)

B

![GFP-PIS](image5)

![LipidTox](image6)

![Merge](image7)
Figure 4. Lipid profiles of CDS knockdown cells

A  
\[ ^{14} \text{C}-\text{Oleate Incorporation} \]

B  
\[ \text{Cellular TAG (Fold Change)} \]

C  
\[ \text{Phospholipids (Fold Change)} \]

Legend:
- siCTRL
- siCDS1
- siCDS2

- **
- *
- ***
Figure 5. PA level upon depletion of CDS

A

Total Cellular PA (Fold Change)

- OA
- +OA

B

High Abundance Species

PA (Fold Change)

PA (16:0)  PA (16:1)  PA (18:0)  PA (18:1)  PA (20:1)  PA (20:2)  PA (27:0)

Low Abundance Species

PA (14:0)  PA (17:0)  PA (26:4)  PA (27:6)  PA (31:0)  PA (33:1)  PA (35:1)  PA (37:2)  PA (43:3)  PA (46:5)

C

Total Cellular PC (Fold Change)

- OA
- +OA

D

High Abundance Species

PC Species (X10^8 Arbitrary Unit)

siCTRL  siCDS1  siCDS2

PC (15:1)  PC (16:0)  PC (16:1)  PC (18:0)  PC (18:1)  PC (33:1)  PC (36:7)

Low Abundance Species

PC (17:0)  PC (17:1)  PC (19:1)  PC (20:0)  PC (31:1)  PC (32:1)  PC (33:1)  PC (34:6)  PC (35:5)  PC (36:3)  PC (38:1)
Figure 5. PA level upon depletion of CDS (Cont.)

E  
Calnexin  
β-actin  
CGI-58

F  

GFP-PASS  Calnexin  Merge

siCTRL  

siCDS1  

siCDS2  

H  
GFP-PASS  MitoTracker  Merge

siCTRL  

siCDS1  

siCDS2  

LD Fractional PA

LD Fractional PC

* *
Figure 6. CDS overexpression inhibits LD expansion

A  
\[ \text{eCFP-N1} + \]  
\[ \text{eCFP-CDS1} + \]  
\[ \text{CDS1} \]  
\[ \text{GAPDH} \]  

B  
\[ \text{CFP} \]  
\[ \text{Nile Red} \]  
\[ \text{Merge} \]  
\[ \text{eCFP-N1} \]  
\[ \text{eCFP-CDS1} \]  
\[ \text{CDS1} \]  
\[ \text{GAPDH} \]  

D  
\[ \text{mCherry-N1} + \]  
\[ \text{CDS2-mCherry} + \]  
\[ \text{RFP} \]  
\[ \text{GAPDH} \]  

E  
\[ \text{mCherry} \]  
\[ \text{BODIPY} \]  
\[ \text{Merge} \]  
\[ \text{mCherry-N1} \]  
\[ \text{CDS2-mCherry} \]  

C  
\[ \text{Diameter (µm)} \]  
\[ \text{% of Lipid Droplets} \]  
\[ \text{Diameter (µm)} \]  
\[ \text{% of Lipid Droplets} \]  

F  
\[ \text{Cellular TAG (Fold Change)} \]  
\[ \text{sCTRL} \]  
\[ \text{sCDS1} \]  
\[ \text{sCDS2} \]  

G  
\[ \text{Cellular TAG (Fold Change)} \]  
\[ \text{sCTRL} \]  
\[ \text{sCDS1} \]  
\[ \text{sCDS2} \]
Figure 7. CDS overexpression inhibits LD expansion in LPIN1 knockdown cells

A

LPIN1 mRNA / β-actin

siCTRL  siLPIN1

***

0.0  0.2  0.4  0.6  0.8  1.0  1.2

B

BODIPY

siCTRL  siLPIN1

C

siLPIN1

O/E CDS1

eCFP-CDS1  Nile Red

O/E CDS2

CDS2-mCherry  BODIPY

D

Cellular TAG (Fold Change)

PA

PC

Phospholipids (Fold Change)

PE

PG

PI

CTRL  siLPIN1  siLPIN1 + O/E CDS1  siLPIN1 + O/E CDS2

*  **  #  ##

E

**  **

*  **  #  ##

**  **
Figure 8. Knocking down CDS1 inhibits adipocyte differentiation

A

Differentiation (Days)

Relative mRNA Level

Caspase3  

CDS1  

CDS2

B

Differentiation (Days)

Relative mRNA Level

CDS1  

PPARγ  

aP2  

C/EBPα

Pref1  

Caspase3

C

siCTRL  

siCDS1

siCTRL  

siCDS1
Figure 8. Knocking down CDS1 inhibits adipocyte differentiation (Cont.)

D 1.5

CDS2

Relative mRNA Level

0.0 0.5 1.0 1.5

0 2 4 6

PPARγ

ap2

1500

0 2 4 6

2000

0 2 4 6

C/EBPα

siCTRL

siCDS2

E

siCTRL

siCDS2

F

siCTRL

siCDS

siCDS2

Tm 2h 6h 18h

p-IRE1α

t-IRE1α

p-ElF2α

t-ElF2α

CHOP

β-actin

38